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Production of microsclerotia of the fungal entomopathogen *Metarhizium anisopliae* and their potential for use as a biocontrol agent for soil-inhabiting insects☆

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ABSTRACT

Microsclerotia (MS), overwintering structures produced by many plant pathogenic fungi, have not been described for *Metarhizium anisopliae*. Three strains of *M. anisopliae* – F52, TM109, and MA1200 – formed MS in shake flask cultures using media with varying carbon concentrations and carbon-to-nitrogen (C:N) ratios. Under the conditions of this study, all strains produced MS, compact hyphal aggregates that become pigmented with culture age, in addition to more typical blastospores and mycelia. While all strains formed desiccation tolerant MS, highest concentrations (2.7–2.9 $\times$ 10^8 L^{-1} liquid medium) were produced in rich media with C:N ratios of 30:1 and 50:1 by strain F52. All three strains of *M. anisopliae* produced similar biomass concentrations when media and growth time were compared. Strain MA1200 produced higher concentrations of blastospores than the other two strains of *M. anisopliae* with highest blastospore concentrations (1.6 and 4.2 $\times$ 10^8 blastospores ml^{-1} on days 4 and 8, respectively) in media with the highest carbon and nitrogen concentrations. Microsclerotial preparations of *M. anisopliae* containing diatomaceous earth survived air-drying (to <5 % moisture) with no significant loss in viability. Rehydration and incubation of air-dried MS granules on water agar plates resulted in hyphal germination and sporogenic germination to produce high concentrations of conidia. Bioassays using soil-incorporated, air-dried MS preparations resulted in significant infection and mortality in larvae of the sugar beet root maggot, *Tetanops myopaeformis*. This is the first report of the production of sclerotial bodies by *M. anisopliae* and provides a novel approach for the control of soil-dwelling insects with this entomopathogenic fungus.

Introduction

For over 60 y, chemical pesticides have been the prevalent tool for insect, weed, and plant disease control. Interest in the use of biologically based pest control measures has been brought about by the development of pest resistance to many chemical pesticides coupled with public concerns about the adverse impact of widespread chemical use on human health, food

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safety, and the environment (Moore & Prior 1993; Auld & Morin 1995). In the late 19th century, Metchnikoff was the first to describe *Metarhizium anisopliae* ''green muscardine'' infections on the cereal cockchafer and to suggest the use of the microorganism as a biological control agent for insects (Zimmermann *et al.* 1995). Subsequent studies showed that an application of *M. anisopliae* conidia could be used to kill the cereal cockchafer and the sugar beet weevil *via* direct infection. Since that time, hundreds of fungi have been identified and are being developed as biological control agents for various insect and weed pests (Copping 2004).

The entomopathogenic fungus *M. anisopliae* has been reported to infect more than 100 insects including a number of soil-dwelling insects: *Reticulitermes* and *Coptotermes* spp., *Diabrotica* spp, *Otiorhynchus sulcatus*, *Diaprepes abbreviatus*, *Popillia japonica*, and *Rhizotrogus majalis* (Krueger *et al.* 1992; Schwarz 1995; Krueger & Roberts 1997; Bruck 2005). The choice of pursuing soil-dwelling insects as targets for biological control *versus* insects on the phylloplane is tempting. The deleterious effects of UV radiation and removal by rainfall are avoided in the soil. Temperatures are moderated in the soil, and soil moistures above the permanent wilting point of plants are well within the optimal range for survival and even growth of many microorganism, especially the fungi. Commercially important targets include root weevils, soil grubs, rootworms, wireworms, fruit flies, and root maggots (Bruck 2005; Chandler & Davidson 2005; Kabaluk *et al.* 2005; Krueger & Roberts 1997; Vanninen *et al.* 1999). Commercial interest in using *M. anisopliae* to control soil-dwelling insects has resulted in pest control formulations based on liquid culture-produced mycelial pellets, encapsulated fungal biomass, or solid substrate-produced conidia, either in aqueous suspension or on a nutritive or non-nutritive granular carrier (Andersche *et al.* 1995; Schwarz 1995; Storey *et al.* 1990).

Infection by this and related entomopathogenic ascomycetes involves the percutaneous penetration into an insect's body from conidia attached to and germinating on the insect's cuticular surface. As a preliminary step, the conidia must contact the insect cuticle in sufficient numbers to infect and kill the host. Thus, conidial concentration and distribution in the soil must be adequate so that insects moving through the soil acquire a fatal dose of conidia (Bruck 2005; Hu & St. Leger 2002; Jaronski 2007). While aqueous or dry suspensions of conidia can be mixed into soil, homogenous distribution through the soil matrix is difficult. A preferred, more practical formulation is a granular preparation, much like the many ''at-planting'' formulations of chemical insecticides. With this approach, numerous foci of large numbers of conidia are created. An insect crawling through the soil needs to encounter one or a few foci (granules) to acquire an infectious and fatal dose of conidia. In addition, granular formulations are also more suitable for standard ''at-planting'' application equipment.

For persistence in soil and decaying plant material, many plant pathogenic fungi produce sclerotia; i.e., compact hyphal aggregates, often melanized, that are highly resistant to desiccation. These propagules serve as the overwintering structure for fungi (Cooke 1983; Coley-Smith & Cooke 1971). Sclerotial bodies have also been shown to be produced by a limited number of entomopathogenic fungi including *Cordyceps*, *Hirsutella*, and *Synnematium* species (Evans & Samson 1982; Speare 1920). Microsclerotia (MS), 200–600 μm diameter sclerotial particles of fungal plant pathogens such as *Colletotrichum truncatum* and *Mycoleptodiscus terrestris* have been produced in high concentration in submerged liquid culture fermentation (Jackson & Schisler 1995; Shearer & Jackson 2003, 2006). For use as bioherbicides, MS of these pathogens of weedy plants have shown value as persistent, infective propagules in soil and in aquatic environments (Boyette *et al.* 2007; Shearer & Jackson 2006).

Our study initially focused on evaluating liquid culture production methods for stable propagules of various strains of the entomopathogenic fungus *M. anisopliae* for use in soil to control the sugar beet root maggot (*Tetanops myopaeformis*). We discovered that the *M. anisopliae* strains under study produced not only blastospores and mycelia, but also melanized MS. We evaluated different liquid culture nutritional environments, measured biomass accumulation, and blastospore and MS yields. Desiccation tolerance was evaluated by air-drying MS and by measuring their ability to germinate hyphally and/or sporogenically upon rehydration. The biocontrol efficacy of soil-incorporated microsclerotia of *M. anisopliae* was evaluated based on their ability to infect and to kill larval sugar beet root maggots in soil.

Materials and methods

Cultures and growth conditions

Three strains of *M. anisopliae* var. *anisopliae* were used in this study: a commercial strain, F52 (ATCC 90448, Novozyme Biologicals, Salem VA), MA1200 (ATCC 62176, originally isolated from *Heterodera glycines* egg cysts), and TM109 (ARSEF5520, originally isolated from *Delia floralis*). All these strains have been under evaluation as mycoinsecticides for the control of *T. myopaeformis* (sugar beet root maggot) and were passaged through larvae of that insect. Each re-isolation was from a single colony and respective strains were cultured twice on agar media before storage at −80 °C.

For liquid culture work, single spore isolates of each strain of *M. anisopliae* were grown on potato dextrose agar (PDA) for three weeks at room temperature. The sporulated agar plate was cut into 1 mm^2 agar plugs and stock cultures of these agar plugs stored in 10 % glycerol at −80 °C. Conidial inocula for liquid culture experiments were produced by inoculating PDA plates with a conidial suspension from the frozen stock cultures and growing these cultures at room temperature (~22 °C) for 2–3 wks. Conidial suspensions were obtained from sporulated PDA plates by rinsing plates with 10 mL of a solution of 0.04 % polyoxyethylene sorbitan mono-oleate (Tween® 80, Sigma). All liquid cultures were inoculated with a conidial suspension of *M. anisopliae* to provide an initial concentration of 5×10^6 conidia ml^{-1} in the culture broth.

The six liquid media tested were composed of a basal salts medium supplemented with trace metals and vitamins (Jackson *et al.* 1997) and various combinations of glucose and acid-hydrolyzed casein (Casamino acids®, Difco Laboratories, Detroit, MI, USA). The defined basal salts solution used in all